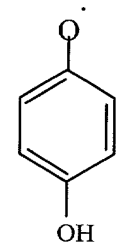
CLAIMS

1) A process for the isolation of p-benzosemiquinone of formula 1



(Formula I)

a major harmful oxidant from cigarette smoke responsible for the oxidative damage of proteins and DNA, the said process comprising the steps of

(a) collecting tar or cs (cigarette smoke) solution from lighted conventional filtered tipped cigarettes,

(b) collecting tar by lighting conventional filter-tipped cigarettes having a tar content of 20 -30 mg per cigarette in a glass flask dipped in a mixture of ice and salt and allowing the tar to condense and settle at the bottom of the flask,

(c) keeping the above said flask at room temperature and extracting the said tar with 30-60 mM potassium phosphate buffer at a pH ranging between 7.4 to 7.8, filtering the above solution through 0.45 μm Millipore filter and adjusting the pH of the filtrate ranging between 7.4 to7.6 by adding NaOH solution to obtain the desired tar solution,

(d) extracting the above said tar solution thrice with equal volume of methylene chloride, discarding the lower methylene chloride layer and collecting the upper yellow coloured aqueous layer termed as aqueous extract of cigarette smoke.

(e) extracting the above said aqueous extract of cigarette smoke twice with equal volume of water saturated n-butanol, lyophilizing the pooled yellow butanol extract in a lyophilizer at a temperature ranging between $-50\,^0$C to -60^0C under vacuum followed by extraction of the lyophilized material twice with HPLC grade acetone and drying the acetone solution under vacuum and dissolving the said acetone extract with HPLC grade methanol,

(f) subjecting the above said methanol solution to band TLC using non-fluorescent silica plates, developing the said silica plates using a mixture of toluene and ethyl acetate in a ratio of 80:20, taking out the said plate and drying it at about 25-30° C using a drier, cutting small strips containing the developed material from both sides of the plates and keeping them in an iodine chamber for the location of the band corresponding to Rf 0.26 , scraping the band and extracting the band material with HPLC grade acetone followed by collection of the acetone layer and drying it under vacuum,

(g) dissolving the above said acetone extract which appeared as pale yellow needles by adding equal volume of milli Q water, extracting the resultant aqueous solution with equal volume of HPLC grade water saturated n-butanol followed by drying upper n-butanol layer in small glass tubes under vacuum to obtain the major cigarette smoke (cs) oxidant with a purity of 98-99% and yield of about 18-22 μg per cigarette,

(h) purifying the above said cs oxidant as obtained in step (g) by dissolving it in a mobile solvent comprising a mixture of methylene chloride and methanol in a ratio of 90:10 (v/v) and injecting it in a HPLC instrument with a normal phase 25 cm silica column using a uv detector at 294 nm at a flow rate of o.5 ml/min, at a temperature of about 25^0C and at a pressure of about 29 kgf/cm^2 followed by collecting the effluent which appears as a single peak at a retention time of 8.808 min with a purity of 100 % and yield of 8.4% of the total cs oxidant present in the parent tar solution.

2. A process for the isolation of p-benzosemiquinone of formula 1, a major harmful oxidant from cigarette smoke responsible for the oxidative damage of proteins and DNA, the said process further comprising

(a) passing the whole cigarette smoke collected from conventional filter tipped cigarette having a tar content of 20 -30 mg per cigarette into 30-60 mM potassium buffer at pH 7.4 –7.8, filtering the above solution through 0.45 μm Millipore filter, adjusting the pH of the filtrate ranging between 7.4 to7.6 by adding NaOH solution to obtain the desired cigarette smoke solution (cs solution);

(b) extracting the above said cs solution thrice with equal volume of methylene chloride, discarding the lower methylene chloride layer and collecting the upper yellow coloured aqueous layer termed as aqueous extract of cigarette smoke;

(c) extracting the above said aqueous layer of cigarette smoke twice with equal volume of water saturated n-butanol , lyophilizing the pooled yellow butanol extract in a Lyolab lyophilizer at a temperature ranging between –50 ^{0}C to -60^{0}C under vacuum followed by extraction of the lyophilized material twice with HPLC grade acetone and drying the acetone solution under vacuum and dissolving the said acetone extract with HPLC grade methanol;

(d) subjecting the above said methanol solution to band TLC using non-fluorescent silica plates, developing the said silica plates using a mixture of toluene and ethyl acetate in a ratio of 80:20, taking out the plate and drying at about 25 ^{o} C to 30 ^{o} C using a drier, cutting small strips containing the developed material from both sides of the plates and keeping them in an iodine chamber for the location of the band corresponding to Rf 0.26 , scraping the band and extracting the band material with HPLC grade acetone followed by collection of the acetone layer and drying it under vacuum;

(e) dissolving the above said acetone extract which appeared as pale yellow needles by adding equal volume of milli Q water , extracting the aqueous solution with equal volume of HPLC grade water saturated n-butanol followed by drying the upper n-butanol layer in small glass tubes under vacuum to obtain the major cs oxidant with a purity of 98-99% and yield of 18-22 μg per cigarette; and

(f) purifying the above said cs oxidant as obtained in step e by dissolving it in a mobile solvent comprising a mixture of methylene chloride and methanol in a ratio of 90:10(v/v) and injecting it in a HPLC instrument with a normal phase 25 cm silica column using a uv detector at 294 nm at a flow rate of o.5 ml/min, at a temperature of about 25^{0}C, at a pressure of about 29 kgf/cm^{2} and collecting the effluent which appears as a single peak at a

retention time of 8.808 min with a purity of 100 % and yield of 8.4% of the total cs oxidant present in the parent cs solution.

3. A process as claimed in claims 1&2 wherein the said isolated pure cigarette smoke (cs) oxidant has the following properties

(a) when crystallized from acetone solution appears as small needle shaped faint yellow coloured crystals having pungent smell, similar to that of rancid butterfat,

(b) UV absorption maxima in methanol solution are at 293.4 nm and 223.0 nm and in aqueous solution are in 288nm and 221nm respectively,

(c) on excitation at 293 nm in methanol solution the observed emission maxima are at 329.6 nm and 651.4 nm and on excitation at 224 nm, the observed emission maxima are at 329.6 nm and 652.6 nm respectively,

(d) when excitation scanning is monitored keeping the emission at 330 nm, the observed excitation maxima are at 228.2 nm and 293.8 nm and when the emission is kept at 651 nm and excitation scanning is monitored, the observed excitation maxima are at 229.2 nm and 294.8 nm respectively,

(e) highly soluble in methanol, ethanol, acetone, n-butanol, fairly soluble in water, sparingly soluble in methylene chloride, di-ethyl ether, chloroform and insoluble in benzene and petroleum ether,

(f) the compound looses its oxidizing potency in acidic pH ranging between 4 to 5 and on keeping the solution at alkaline pH ranging between 9 to 10, the compound gradually turns brown, at pH 10 and above there is instantaneous darkening with loss of both activity and aromaticity as evidenced by UV spectroscopy,

(g) the half-life of the oxidant, when stored in the solid state at a temperature ranging between 25 °C to 30 °C under darkness is about 48 hours as determined by its oxidative potency, but in solution of 50 mM potassium phosphate buffer, pH 7.4 at 25°C to 30°C the half life is about 1hour 30 min,

(h) reduces ferricytochrome c and ferric chloride,

(i) oxidizes ascorbic acid , proteins and DNA, and

(j) the melting point is 162°C,

4. A process for the quantitative determination of p-benzosemiquinone of formula 1, a major harmful oxidant isolated from cigarette smoke responsible for the oxidative damage of proteins and DNA, the said process comprising the steps of

   (a) collecting tar or cs (cigarette smoke) solution from lighted conventional filtered tipped cigarettes,

   (b) collecting tar by lighting conventional filter-tipped cigarettes having a tar content of 20 -30 mg per cigarette in a glass flask dipped in a mixture of ice and salt and allowing the tar to condense and settle at the bottom of the flask,

   (c) keeping the above said flask at room temperature and extracting the said tar with 30-60 mM potassium phosphate buffer at a pH ranging between 7.4 to 7.8, filtering the above solution through 0.45 μm Millipore filter and adjusting the pH of the filtrate ranging between 7.4 to7.6 by adding NaOH solution to obtain the desired tar solution,

   (d) extracting the above said tar solution thrice with equal volume of methylene chloride, discarding the lower methylene chloride layer and collecting the upper yellow coloured aqueous layer termed as aqueous extract of cigarette smoke,

   (e) extracting the above said aqueous extract of cigarette smoke twice with equal volume of water saturated n-butanol, lyophilizing the pooled yellow butanol extract in a lyophilizer at a temperature ranging between $-50\,^{0}C$ to $-60^{0}C$ under vacuum followed by extraction of the lyophilized material twice with HPLC grade acetone and drying the acetone solution under vacuum and dissolving the said acetone extract with HPLC grade methanol,

   (f) subjecting the above said methanol solution to band TLC using non-fluorescent silica plates, developing the said silica plates using a mixture of toluene and ethyl acetate in a ratio of 80:20, taking out the said plate and drying it at about 25-30 ^{o} C using a drier followed by cutting small strips containing the developed material from both sides of the plates and keeping them in an iodine chamber for the location of the band corresponding to Rf 0.26 , scraping the band and extracting the band material with HPLC grade acetone followed by collection of the acetone layer and drying it under vacuum,

(g) dissolving the above said acetone extract which appeared as pale yellow needles by adding equal volume of milli Q water, extracting the resultant aqueous solution with equal volume of HPLC grade water saturated n-butanol followed by drying upper n-butanol layer in small glass tubes under vacuum to obtain the major cigarette smoke (cs) oxidant with a purity of 98-99% and yield of about 18-22 μg per cigarette, and

(h) purifying the above said cs oxidant as obtained in step (g) by dissolving it in a mobile solvent comprising a mixture of methylene chloride and methanol in a ratio of 90:10 (v/v) and injecting it in a HPLC instrument with a normal phase 25 cm silica column using a uv detector at 294 nm at a flow rate of o.5 ml/min, at a temperature of about 25^0C and at a pressure of about 29 kgf/cm^2 followed by collecting the effluent which appears as a single peak at a retention time of 8.808 min with a purity of 100 % and yield of 8.4% of the total cs oxidant present in the parent tar solution.

5. A process for the quantitative determination of p-benzosemiquinone of formula 1, a major harmful oxidant isolated from cigarette smoke responsible for the oxidative damage of proteins and DNA, the said process further comprising

(a) passing the whole cigarette smoke collected from conventional filter tipped cigarette having a tar content of 20 -30 mg per cigarette into 30-60 mM potassium buffer at pH 7.4 –7.8, filtering the above solution through 0.45 μm Millipore filter, adjusting the pH of the filtrate ranging between 7.4 to7.6 by adding NaOH solution to obtain the desired cigarette smoke solution (cs solution),

(b) extracting the above said cs solution thrice with equal volume of methylene chloride, discarding the lower methylene chloride layer and collecting the upper yellow colored aqueous layer termed as aqueous extract of cigarette smoke,

(c) extracting the above said aqueous layer of cigarette smoke twice with equal volume of water saturated n-butanol , lyophilizing the pooled yellow butanol extract in a Lyolab lyophilizer at a temperature ranging between –50 ^{0}C to -60^0C under vacuum followed by extraction of the lyophilized material twice with HPLC grade acetone and drying the acetone solution

under vacuum and dissolving the said acetone extract with HPLC grade methanol,

(d) subjecting the above said methanol solution to band TLC using non-fluorescent silica plates, developing the said silica plates using a mixture of toluene and ethyl acetate in a ratio of 80:20, taking out the plate and drying at about 25 ^{o} C to 30 ^{o} C using a drier, cutting small strips containing the developed material from both sides of the plates and keeping them in an iodine chamber for the location of the band corresponding to Rf 0.26 , scraping the band and extracting the band material with HPLC grade acetone followed by collection of the acetone layer and drying it under vacuum,

(e) dissolving the above said acetone extract which appeared as pale yellow needles by adding equal volume of milli Q water , extracting the aqueous solution with equal volume of HPLC grade water saturated n-butanol followed by drying the upper n-butanol layer in small glass tubes under vacuum to obtain the major cs oxidant with a purity of 98-99% and yield of 18-22 µg per cigarette, and

(f) purifying the above said cs oxidant as obtained in step e by dissolving it in a mobile solvent comprising a mixture of methylene chloride and methanol in a ratio of 90:10(v/v) and injecting it in a HPLC instrument with a normal phase 25 cm silica column using a uv detector at 294 nm at a flow rate of o.5 ml/min, at a temperature of about 25^{0}C , at a pressure of about 29 kgf/cm^{2} and collecting the effluent which appears as a single peak at a retention time of 8.808 min with a purity of 100 % and yield of 8.4% of the total cs oxidant present in the parent cs solution.

6. A process as claimed in claims 1 to 4, wherein p-benzosemiquinone present in cs solution is quantitatively assayed by HPLC with a UV detector using a 25 cm reverse phase ODS column and using a mixture of water and methanol (95: 5 v/v) as a mobile phase, at a wave length of 288nm, flow rate of 0.8 ml/min, at a temperature of about 25°C and at a pressure of about 147 Kgf/cm^{2} and having a retention time of 13.46 min.

7. A process as claimed in claim 1, wherein the said p-benzosemiquinone is responsible for the major cause of oxidative damage of proteins isolated from the whole cs solution.

8. A process as claimed in claim 1, wherein p-benzosemiquinone, the cs oxidant is responsible for the oxidative damage of DNA.

9. A process as claimed in claim 1, wherein the damage of proteins caused by p-benzosemiquinone present in cs solution is quantitatively determined by measuring protein carbonyl formation by reacting the protein with p-benzosemiquinone obtained from the cs solution, followed by reaction with 2,4 dinitrophenyl hydrazine (DNPH) and finally measuring the absorbance at a wave length of 390nm.

10. A process as claimed in claim 1, wherein the damage of proteins caused by p-benzosemiquinone present in cs solution is quantitatively determined by measuring oxidative degradation of guinea pig tissue microsomal proteins by reacting the said protein with p-benzosemiquinone present in cs solution followed by SDS-PAGE and densitometric scanning.

11. A process as claimed in claim 10, wherein the protein used for the assay of oxidative damages of protein is selected from the group consisting of BSA and guinea pig lung microsomal proteins.

12. A process as claimed in claim 10, wherein the BSA oxidation produced by the whole cs solution is effected by the p-benzosemiquinone present in the cs solution.

13. A process as claimed in claim 12, wherein the BSA oxidation produced by the cs oxidant as evidenced by nmoles of carbonyl formed per mg BSA is 9.56 ± 0.14 in comparison to 7.53 ± 0.34 produced by the whole cs solution.

14. A process as claimed in claim 12, wherein the BSA oxidation produced by the cs oxidant as evidenced by nmoles of carbonyl formed per mg BSA is 9.56 ± 0.14 in comparison to 8.16 ± 0.24 produces by the aqueous extract of cigarette smoke.

15. A process as claimed in claim 12, wherein the BSA oxidation produced by the cs oxidant as evidenced by nmoles of carbonyl formed per mg BSA is 9.56 ± 0.14 in comparison to 9.23 ± 0.14 produces by the TLC purified aqueous extract of cigarette smoke.

16. A process as claimed in claim 11, wherein the oxidative degradation of guinea pig tissue microsomal proteins produced by the p-benzosemiquinone solution as evidenced by SDS-PAGE is comparable to that produced by the whole cs solution.

17. A process as claimed in claim 1, wherein the said method is used for quantitative determination of cs oxidant p-benzosemiquinone in cigarettes based on the tar content of the particular commercial brand of the cigarette.

18. A process as claimed in claim 1, wherein the said method is used for quantitative determination of cs oxidant p-benzosemiquinone in cigarettes based on toxicity level of the particular commercial brand of the cigarette.

19. A process as claimed in claim 1, wherein the amount p-benzosemiquinone isolated from smoke of different commercial brands of burning cigarettes is used to determine the toxicity index of a particular brand of cigarette based on the quantity of p-benzosemiquinone present.

20. A method for the prevention of cigarette smoke induced protein oxidation in vitro, said method comprises inhibiting the BSA oxidation by using a chemical compound or agent selected from the group consisting of ascorbic acid, sodium dithionite, tartaric acid, citric acid, oxalic acid, succinic acid, histidine, lysine, thiourea, glutathione, black tea extract, green tea extract, catechin, epigallocatechin and epicatechin.

21. A method as claimed in claim 20 wherein ascorbic acid inhibits BSA oxidation up to 76% at a concentration of about 100 μM.

22. A method as claimed in claim 20 wherein Sodium dithionite inhibits BSA oxidation up to 97% at a concentration of about 2 mM.

23. A method as claimed in claim 20 wherein tartaric acid inhibits BSA oxidation up to 75% at a concentration ranging between 500 μM to 1 mM.

24. A method as claimed in claim 20 wherein citric acid inhibits BSA oxidation up to 75% at a concentration ranging between 500 μM to 1 mM.

25. A method as claimed in claim 20 wherein oxalic acid inhibits BSA oxidation up to 53% at a concentration of about 500 μM.

26. A method as claimed in claim 20 wherein succinic acid inhibits BSA oxidation up to 60% at a concentration of about 1mM.

27. A method as claimed in claim 20 wherein histidine acid inhibits BSA oxidation up to 67% at a concentration of about 1mM.

28. A method as claimed in claim 20 wherein black tea extract inhibits BSA oxidation up to 50% at a concentration of about 2.5mg.

29. A method as claimed in claim 20 wherein catechin inhibits BSA oxidation up to 54% at a concentration of about 750 μg.

30. A method as claimed in claim 20 wherein epigallocatechin inhibits BSA oxidation up to 95% at a concentration of about 140 μg.

31. A method as claimed in claim 20 wherein epicatechin inhibits BSA oxidation up to 50% at a concentration of about 50 μg.

32. A method as claimed in claim 20 wherein green tea extract inhibits BSA oxidation up to 50% at a concentration of about 2.5mg.

33. A method as claimed in claim 20 wherein lysine inhibits BSA oxidation up to 35% at a concentration of about 1mM.

34. A method as claimed in claim 20 wherein thiourea inhibits BSA oxidation up to 52% at a concentration of about 10mM.

35. A method as claimed in claim 20 wherein glutanthione inhibits BSA oxidation up to 37% at a concentration of about 1mM.

36. Use of chemical compounds or agents selected from the group consisting of ascorbic acid, sodium dithionite, tartaric acid, citric acid, oxalic acid, succinic acid, histidine, lysine, thiourea, glutathione, black tea extract, green tea extract, catechine, epigallocatechin and epicatechin as a antidote for the harmful effect caused by the cigarette smoke oxidant.

37. Use of the compound p-benzosemiquinone for studying the mechanism of oxidative damage-induced degenerative diseases and cancer caused by cigarette smoke producing oxidative damage to isolated protein, DNA, cultured cells or to an experimental model under laboratory conditions.

38. A method for quantitative estimation of an harmful oxidant, p-benzosemiquinone, the said method is helpful in formulating the quantity and nature of smoking material to be used in cigarette, cigar, cigarette pipes and any other convention smoking devices.